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Sensitivity and Specificity of Cocaine Metabolite Screening in View of the Analytical Performance of a Fluorescence Polarisation Immunoassay

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Dedicated to Prof. Dr. Dr. h. c. Helmut Wachter, Innsbruck, on the occasion of his 65th birthday

Summary: Immunoassays for drug screening are called 'qualitative' or 'semiquantitative' by the manufacturers of these tests and they urgently recommend the user to verify each result exceeding the recommended cut-off value by a confirmation test. For therapeutic drug monitoring assays or for the determination of tumour markers or hormones, similar recommendations are not given, although the same analytical technologies are used for these assays.

A scientific validation of the commonly used cut-off values recommended by the official bodies (e. g. NIDA, DoD) is unfortunately not described in the accessible scientific literature.

A solution to this problem was sought by evaluating the analytical characteristics of the assay and determining the diagnostic validity of the test using an immunoassay for the cocaine metabolite as an example.

Hundred urine samples from people suspected of cocaine abuse and 50 urine samples from patients unlikely to have consumed cocaine were analysed in triplicate with a commercially available fluorescence polarisation immunoassay. From this data we assessed the analytical variance of the assay using the computer program of *Sadler & Smith* (Clin. Chem. *36* (1990), 1346–1350). Using the functions provided, we calculated the limit of detection (LD) and the lower limit of quantification (LLQ) as well as the so-called power of definition (PD) using a recently published method (*Gautschi* et al., this journal *31* (1993), 433–440). This procedure is mathematically well defined, uses no artificial standards or calibrators and is in compliance with IFCC recommendations.

A clearly defined assessment of the diagnostic performance of an assay is of utmost importance for the discussion of adequate decision levels. The influence of different decision levels was demonstrated by assessing the diagnostic performance of the FPIA assay for benzoylecgonine by calculating the predictive values of a negative and positive test result for four different decision limits (12, 40, 150, recommended by DoD and 300 µg/l, recommended by NIDA). The respective predictive values of the negative results were 0.931, 0.864, 0.704 and 0.661. The predictive value of a positive test result was uniformly 1.0 for all four cut-offs.

These results are critically discussed with respect to the analytical performance of the assay, the socio-economic and legal consequences of the screening procedure.

Introduction

There are several reasons for screening people for drug abuse. The primary uses are however, surveillance of former drug addicts taking part in drug abuse rehabilitation programs, routine 'on the job' or pre-employment screening and testing people with unexplained, abnormal behaviour.

During the past few years, the number of samples submitted for drug screening has increased dramatically in our institution and can constitute up to 350 urine sam-

ples a day. New methodological approaches had to be evaluated in order to guarantee a rapid turnover as well as a reliable performance of the methods used in drug testing. The obligation to confirm the increasing number of positive results by an alternative (reference) method (e. g. GC-MS) requires additional personnel and increases costs accordingly.

Modern immunological tests designed for drug screening using highly automated systems (e. g. EMIT II, Syva; TDx, Abbott) are described as being 'semiquantitative' in the method descriptions supplied by the manufacturers, without giving detailed information on the assay performance and how the data given (e. g. limit of detection) were obtained.

Drug testing is a 'forensic toxicological' analysis, and as *Peat* stated: "... this requires a strict chain of custody, security of the laboratory, appropriate and adequate quality control procedures, and the use of legally defensible analytical procedures (1)". However, it is astonishing that for tests whose results have such far-reaching consequences (false negative as well as false positive results), the performance of the assays, especially the limit of detection, the limit of quantification and the threshold concentration have not been evaluated in studies by using standardized and acceptable mathematical procedures.

It has been known for many years that the imprecision of an assay depends upon the concentration of the analyte, but it was not until the introduction of radioimmunoassays that thorough studies have been published on the issue (2). Further publications have shown that this observation is also valid for other procedures (3). At each analyte concentration a specific imprecision must therefore be expected and general data cannot be given.

Despite the fact that several authors (2−4) have proposed the derivation of the analytical limit from a precision profile, most method descriptions or evaluations use procedures which are statistically not clearly defined. To our knowledge, no standardized mathematical approaches have been used until now to assess the performance of immunoassays for drugs of abuse.

A standardized mathematical procedure has been described in the literature for the evaluation of cyclosporin (3) and prostate specific antigen (5). Data derived from human sample material was used to determine the variance function, which was subsequently applied to determine the analytical limits. No artificial standard material with a matrix different from human samples had to be used. The characteristics which could be derived from the variance function were:

(I) the precision profile,
(II) the limit of detection (LD) and
(III) the limits of quantification (LLQ, ULQ).

Additionally, the power of definition (PD) can be calculated for every interval of interest (3, 5). The PD allows one to determine which differences in subsequent values represent significant (with $p > 0.95$) changes at each defined interval of the analytical range.

In this paper we applied a standardized mathematical procedure for the assessment of the performance of the cocaine metabolite (benzoylecgonine) assay, which is based on a fluorescence polarisation immunoassay.

The term 'analytical sensitivity' of the method is critically discussed both in relation to the consequences of the definition of the cut-off at different levels and with respect to the diagnostic sensitivity and specificity of the screening procedure for the abuse of cocaine. An alternative procedure for the definition of the threshold value is proposed.

Materials and Methods

Hundred urine samples of people suspected of cocaine abuse when entering a drug rehabilitation centre ('patients' group) were analyzed in triplicates with the Abbott TDx (fluorescence polarisation immunoassay, FPIA; Abbott Laboratories, North Chicago). In addition 50 urine samples from patients from the intensive care unit were included in the study. These patients were being treated with several different therapeutic drugs, but an abuse of cocaine could be excluded ('control' group).

The FPIA-method and the instrument technology has been described in detail elsewhere (6). The TDx uses a six-point calibration curve and prints out quantitative results.

In order to calculate the diagnostic sensitivity and specificity of the procedure, the test results of these two patient groups were attributed to 13 different classes from 1.6 to ≥ 400 µg/l and plotted using the computer program of *Abendroth* et al. (7). Diagnostic sensitivity, diagnostic specificity and negative and positive predictive values of the screening procedure for the cocaine metabolite were calculated for four different cut-off values.

The variance function and the corresponding confidence interval were derived from triplicate measurements of patient samples using the computer-program by *Sadler & Smith* (8). The analytical variance can be assessed with a minimum of duplicate measurements. By increasing the number of repetitions, the meaning of each individual coefficient of variation (CV) can be increased. Triplicate measurements were chosen as a compromise between the need to obtain a meaningful CV for every sample and the need to limit the reagent costs for such a study.

The calculation of the precision profile, the lower limit of quantification (LLQ), the limit of detection (LD) and the power of definition (PD) has been described previously (3). In short: the precision profile (CV versus analyte concentration) can be derived from the variance function. The intersection of a horizontal line − corresponding to an acceptable CV of the method − with the upper limit of the confidence interval of the precision profile represents the lower limit of the quantification interval (LLQ) [For details see figure 2 (inset)].

By setting the analyte concentration (U) to zero in the variance function, the standard deviation of the blank can be extrapolated

and a normal distribution with the respective standard deviation can be constructed. Using an iterative mathematical procedure (*Newton-Rawson*-procedure (9)) the adjacent normal distribution with a defined overlap of 5% is constructed. The peak value of this second normal distribution represents the LD. The intersection of the two normal distributions represent the critical limit (LC) (fig. 3).

In order to determine the PD for each interval of interest, a series of normal distributions can be constructed, each overlapping the previous by e. g. 5%. The PD is a measure for the 'analytical sensitivity' of the method for a distinct interval. The programs for the calculation of LC, LD, LLQ and PD were developed using the computer program 'Microsoft® Excel, version 4.0'.

Results

A. Analytical performance of the cocaine metabolite asssay

Using the PC-program by *Sadler & Smith* (8) the *variance function* could be assessed, which describes the dependence of the variance on the concentration of the analyte (fig. 1). The function runs as follows: s^2 (U) = (2.050 + 0.0048 (U))$^{3.028}$. From this variance function the precision profile could be derived, as shown in figure 2.

The determination of the *lower limit of quantification* (LLQ) was performed according to the procedure shown in the enlargement in figure 2. The LLQ was determined to be 39 µg/l (10% CV) or 92 µg/l (5% CV). The upper limit of quantification for this assay is given by the concentration of the highest calibrator (1000 µg/l) and not by the performance of the assay, since the CV at an analyte concentration of 1000 µg/l is 1.8%.

The coefficients of variation at analyte concentrations that will be discussed as potential cut-off limits were calculated to be: 25.8% (12 µg/l); 8.5% (40 µg/l); 3.1% (150 µg/l); 2.2% (300 µg/l).

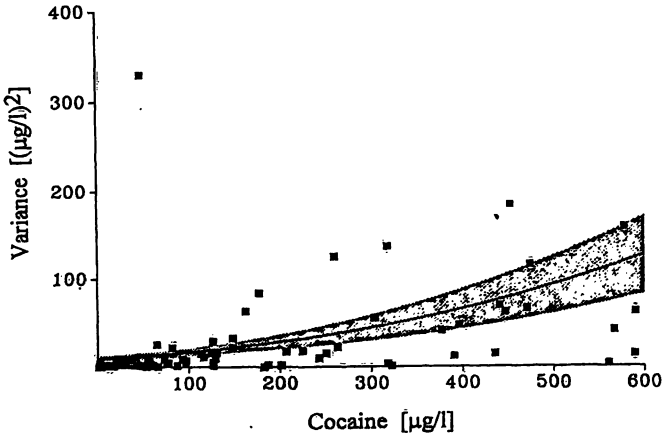

Fig. 1 Analytical variance of triplicate measurements in dependence of the concentration of the analyte (■). The corresponding regression curve and the confidence limits were calculated and plotted by the *Sadler-Smith* program (8).

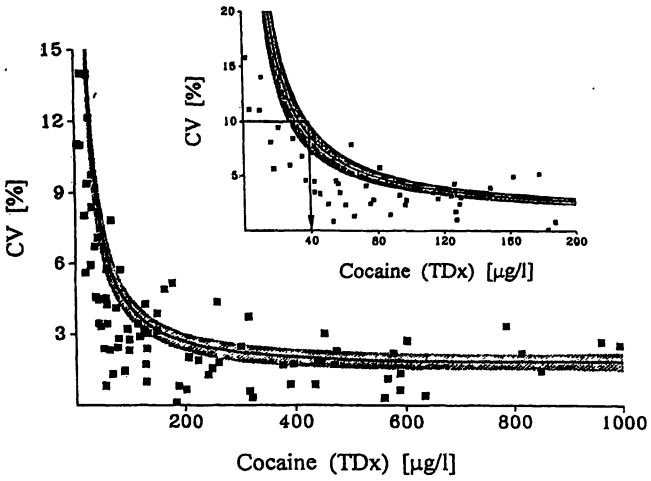

Fig. 2 Precision profile corresponding to the plot of the analytical variance in fig. 1. In the blow up, the determination of the lower limit of quantification (LLQ) is shown.

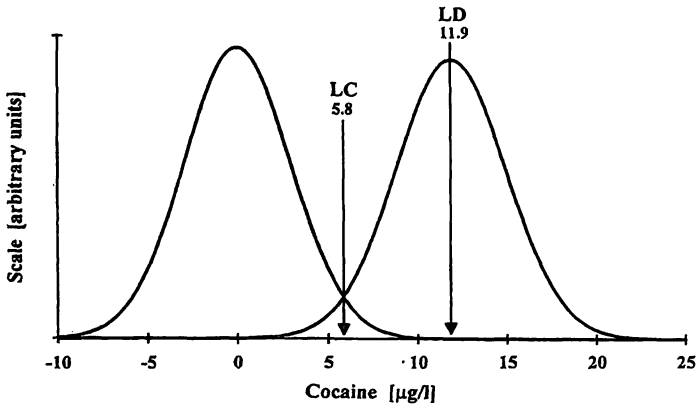

Fig. 3 Construction of the normal distribution of the blank and the adjacent normal distribution following the variance formula and overlapping the zero-distribution by 5%.

The standard deviation of the assay at a benzoylecgonine concentration of zero was calculated by the variance function (s(0) = 2.96) and used for the construction of a normal distribution at an analyte concentration of zero (fig. 3). The intersection of the adjacent normal distribution, overlapping the 'blank normal distribution' by 5%, represents the *critical limit* (LC). At this point the probability for the presence of the analyte equals the probability for the absence of the analyte. It was found to be 5.8 µg/l. The *limit of detection* (LD), where the probability for the detection of the analyte is ≥ 95%, is represented by the peak value of the second normal distribution. LD was found to be 11.9 µg/l. The CV at this concentration is 26.0%.

For every interval of the analytical range, consecutive normal distributions, each overlapping the preceeding by 5%, can be constructed (fig. 4). The number of normal distributions, which equals the *power of definition (PD)*, represents the number of benzoylecgonine values which can be discriminated with a probability of ≥ 95%

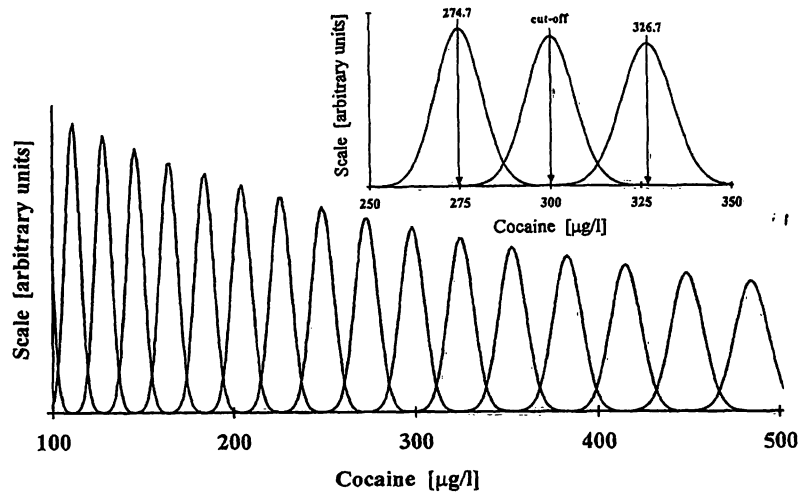

Fig. 4 Assessment of the power of definition (PD) in the interval of 100 µg/l to 500 µg/l. For details see text.

in a certain interval. In the interval 100–500 µg/l, the PD is 16, in the interval 400–500 µg/l, the PD was found to be only = 3.

The power of definition at the cut-off value (according to NIDA = 300 µg/l (10)) is shown in the enlarged section of figure 4. The values which can be discriminated from 300 µg/l with $p \geq 95\%$ are ≤ 274.7 µg/l or ≥ 326.7 µg/l.

B. Diagnostic performance of the cocaine metabolite screening

The benzyolecgonine values found for the 50 samples from patients of the intensive care unit who were under treatment for a wide spectrum of different therapeutic drugs (population of the 'non-diseased') ranged from 0.0 to 9.8 µg/l, whereas in the cocain addicted population ('diseased') values from 0 µg/l to 586 mg/l were observed. The distribution of the values in the two populations is plotted in figure 5. Sensitivity, specificity and positive and negative predictive values were calculated for four different cut-off values (tab. 1).

The diagnostic sensitivity of the screening procedure is highest (0.926) with a cut-off close to the limit of detection of the method and decreases dramatically to less than 0.5 (0.488) with the cut-off recommended by NIDA (300 µg/l).

With none of the cut-offs used for further calculations, a false positive classification of a patient resulted and the specificity of the screening was therefore uniformly = 1.0.

A consequence of the 100% specificity of the screening is that the predictive value of the positive result is uniformly = 1.0. The predictive value of the negative result depends on the diagnostic sensitivity of the procedure and on the prevalence of the drug abuse. Predictive values of the negative result for an assumed prevalence of 0.5 are given in table 1.

Discussion

Immunological tests for the quantification of drugs of abuse were first introduced as radioimmunoassays in 1970 for the determination of morphine in urine (11). A large scale screening was made possible in many laboratories only when non-radioactive immunoassays for drug testing became commercially available and the degree of automation could be significantly improved. With the rapid technological improvements and the advances in automation technology, the detection limits for the analytes decreased simultaneously. The detection

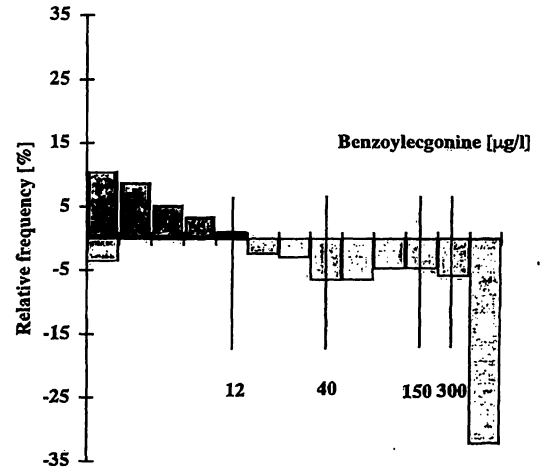

Fig. 5 Distribution of the cocaine metabolite concentrations in urine samples of 50 patients of the intensive care unit (filled bars) and of 100 samples from out-patient clinics for drug addicts (open bars). The consequences of the choice of different cut-off values are summarized in tab. 1. Benzoylecgonine concentrations are given in a logarithmic scale.

Tab. 1 Diagnostic sensitivity, specificity and predictive values for the positive and negative result calculated from the data given in fig. 5 (prevalence = 0.5).

Cut-off (µg/l)	Sensitivity	Specificity	Predictive value of the positive result	Predictive value of the negative result
12	0.926	1.0	1.0	0.931
40	0.843	1.0	1.0	0.864
150	0.579	1.0	1.0	0.704
300	0.488	1.0	1.0	0.661

limits reported for the first immunoassays for the quantification of cocaine in urine were high (RIA: 0.75 mg/l; EMIT-dau: 1.6 mg/l; EMIT-st: 0.75 mg/l) (12), whereas recently issued immunoassays are much more sensitive. For the FPIA method e. g., a LD of 0.03 mg/l has been reported (13). By modifying the calibration procedure of the Abbott TDx method for the cocaine metabolite a decrease of the LD to 0.027 mg/l could be achieved (14).

The antibody used in the cocaine metabolite assay from Abbott is highly specific for benzoylecgonine, the primary urinary metabolite of cocaine. Cross reactivity has to be expected for cocaine (0.4−0.8%), ecgonine (0.3%) and ecgonine methylester (< 0.1% (13)). Only a few substances unrelated to cocaine may cause significant positive interference with immunoassays for benzoylecgonine, e. g. phenothiazines (chlorpromazine and thioridazine) and tricyclic antidepressants (amitriptyline). But these drugs must be present at unlikely concentrations on the order of 100 mg/l (15−17).

If this model were to be used for group-specific tests (e. g. benzodiazepines) one or more compounds (metabolites) must be selected to assess the performance of the assay. The analytical result could be related to a major component for which the antibody used in the test is specific.

We assessed the analytical performance of the FPIA assay using a standardized mathematical procedure for the determination of the variance function. This procedure is based on human sample material. No standards with artificial matrices had to be used. The main problem in estimating the limit of detection lies in obtaining an adequate blank (18). This problem can be elegantly solved by extrapolating the variance function to zero. From the variance function the critical limit (LC), the limit of detection (LD) and the lower (and upper) limit of quantification (LLQ) can be derived according to a procedure which has been previously described in detail (3, 5, 19). This procedure is very compatible with IFCC recommendations (20), which define the limit of detection as a quantity which should be set where "the value can be distinguished from the blank with a stated probability".

The performance of an analytical method whose results may have such severe consequences (not getting a job, loss of job, etc.) should, in our opinion, be assessed using a highly approved and standardized mathematical procedure.

Most of the authors and reagent manufacturers who report on the limit of detection either do not describe the method used to obtain the LD and/or use artificial standard material for its determination.

Limits of detection calculated from repetitive measurements of a 'zero-calibrator' by adding 2 standard deviations (21) or 3 standard deviations (22) to the mean (obtained by a repetitive measurement of a 'blank' sample), in our terminology rather represent the critical limit, where the probability for a false positive estimate is e. g. ≈ 5% (2 s) or ≈ 1% (3 s). Repetitive measurements of the zero calibrator deliver at best an imprecision of the 'system noise', which might follow a normal distribution. Limits based on a certain standard deviation of these measurements therefore represent no more than an exclusion criterion.

In our approach, the limit of detection was set where the probability for detection of the analyte is ≥ 95% (fig. 3). It is, however, important to mention that results in the range of the LD are still burdened with a high imprecision (26.0% at LD). It is therefore necessary to define a CV which is acceptable from a diagnostic point of view and hence to determine the lower limit of quantification, which is significantly different from the LD (fig. 2).

Most results from drug screening are not given as quantitative values, but rather in a nominal form as 'positive' or 'negative'. The decision limit (cut-off) used by most laboratories to obtain this binary result is based on NIDA specifications originally issued in 1988 (10). These cut-off recommendations have not been altered, despite the fact that detectability (often falsely called 'sensitivity') and specificity of the test systems currently used have been substantially improved.

As stated by *Fuentes-Arderiu*, the term 'sensitivity' is "... most confusing in clinical chemistry ..." (18). The majority of authors use it when referring to the limit of detection, in contradiction to international metrological organizations. According to ISO (23), analytical sensitivity is "... the change in response of a measuring instrument divided by the corresponding change in stimulus ...". The same is claimed in other words by IUPAC (24): "... sensitivity is the slope of the analytical calibra-

tion curve ...". Sensitivity defined according to these recommendations is of great importance for test design, but useless for test applications under routine conditions. Here the power of definition is of crucial benefit, because it includes both the slope of the calibration curve (the true sensitivity) and the corresponding variability, e. g. as confidence intervals.

Recommendations issued by the United States Department of Defense in 1993 (directive 1010.1) (25) take the analytical improvement at least partly into account and suggest that the cut-off for the cocaine metabolite assay be lowered to 150 µg/l. The level of the chosen cut-off has a substantial impact on the diagnostic sensitivity of the screening procedure, as shown in figure 5 and table 1. *Finkle* et al. stated that the threshold concentrations are selected on the basis of 'administrative' and 'programmatic' needs (26). In our view, the purpose of screening for an abuse of drugs should be to detect as many recent drug abuses as possible. The results shown in table 1 demonstrate that the diagnostic sensitivity increases substantially if the cut-off is moved to lower values. By lowering the cut-off to 40 µg/l (which is close to LLQ, if a CV of 10% is accepted) a diagnostic sensitivity of 0.843 could be achieved and the predictive value of the negative result could be improved to 0.864 (in a collective with a prevalence for an abuse of cocaine of 0.50) while the diagnostic specificity and the predictive value of the positive result would remain unchanged (= 1.0).

As suggested by others (19, 25), we are convinced that decision levels for the screening for an abuse of cocaine should be lowered. *Hallbach & Guder* proposed '... to use the detection limit as decision limit ...' (27). Taking the usually high imprecision of LD into account, we would recommend the use of LLQ as the decision limit (cut-off).

Modern analytical systems for drug screenings are based on the same principles as assays for the determination of hormones, tumour markers and therapeutic drugs. All of these assays are established as true quantitative methods for diagnostic purposes. Nevertheless, the manufacturers label their drug-screening assays as 'qualitative' or even as 'semiquantitative' tests, as is usually done for urine test strips. But as stated by *Dybkaer* (28) "... these terms are ambiguous and give insufficient information ..." and should therefore be avoided.

Very much care is taken to avoid false positive test results. The users of the test kits are urged to confirm each positive result by a so called 'confirmation test', despite the fact that imprecision as well as detectability of the recommended analytical systems (GC, HPLC) is in many cases worse compared to modern immunological systems, especially in the case of the cocaine metabolite assay where the immunological test is highly specific (29). The medical or forensic screening of (suspected) drug addicts involves a special difficulty in so far as if an abuse of drugs is stated, portentous psychological, social, economical and judicial consequences might result. False results (especially false positives) may even involve the liability for compensation. This might explain why manufacturers of systems for the screening for drugs of abuse advise caution when interpreting test results and do not object to the use of outdated cut-off values.

But it seems to be important to emphasise the fact that official documents urge drug testing laboratories not only to minimize their number of false positive test results by using confirmation procedures, but also state that the number of false negative results has to be kept ≤ 10% (10).

The data presented here demonstrate that – using the NIDA cut-off value – almost half of the urine samples with benzoylecgonine concentrations well above LD are classified as 'negative'. This kind of false negative results can only be minimized if the decision limit is considerably lowered and adapted to the actual analytical state of the art.

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